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Failure of a *Mycobacterium tuberculosis* Δ*RD1* Δ*panCD* double deletion mutant in a neonatal calf aerosol *M. bovis* challenge model: Comparisons to responses elicited by *M. bovis* bacille Calmette Guerin

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Abstract

An attenuated *Mycobacterium tuberculosis RD1* knockout and pantothenate auxotroph (mc^{2}6030) vaccine administered at 2 weeks of age failed to protect calves from low dose, aerosol *M. bovis* challenge at 2.5 months of age. In contrast, *M. bovis* bacille Calmette Guerin (BCG)-vaccinates had reduced tuberculosis-associated pathology as compared to non- and mc^{2}6030-vaccinates. Mycobacterial colonization was not impacted by vaccination. Positive prognostic indicators associated with reduced pathology in the BCG-vaccinated group were decreased antigen induced IFN-γ, iNOS, IL-4, and MIP1-α responses, increased antigen induced FoxP3 expression, and a diminished activation phenotype (i.e., ↓CD25+ and CD44+ cells and ↑CD62L+ cells) in mycobacterial-stimulated mononuclear cell cultures. The calf sensitization and challenge model provides an informative screen for candidate tuberculosis vaccines before their evaluation in costly non-human, primates.
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1. Introduction

Tuberculosis (TB) vaccines for eventual use in humans are typically screened for safety and efficacy using mouse (including immune deficient strains for safety assurance) and guinea pig models of infection [1]. The most promising candidates are evaluated further in non-human primates [2]. A key target population for TB vaccination is the newborn child, particularly in poverty stricken countries where nutritional, respiratory, and enteric diseases are common [3]. Neonatal cynomolgus macaques (*Macaca fascicularis*), while particularly relevant TB models, are costly to house in biosafety level-3 (BL-3) facilities and are limited in availability due to a monestrous reproductive cycle.

Another option for neonatal testing of candidate TB vaccines is the neonatal calf (*Bos taurus*) [4–6]. Large numbers of age-, sex- and breed-matched calves can be acquired throughout the year and calves are substantially less expensive to purchase and house than neonatal monkeys. Because cattle are out-bred, experimental variance is similar to that observed in non-human primates and humans. Additionally, the size of the newborn calf allows dose titration studies at a range relevant to studies in humans. Collection of large volumes of

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blood at frequent intervals is also an advantage of this model. Finally, the nutritional status, that can affect vaccine efficacy, can be manipulated in the neonatal calf to achieve degrees of protein-energy malnutrition [7] comparable to those experienced by human infants in developing countries.

Mycobacterium bovis, a member of the *M. tuberculosis* (*tb*) complex is the most frequently employed challenge strain in cattle studies. In addition to the serious economic and health concerns due to bovine TB, *M. bovis* is increasingly recognized as a major cause of disease in developing nations [8]. Recent TB outbreaks in several US cities are linked with ingestion of *M. bovis*-infected, non-pasteurized cheese from Mexico [9]. Thus, efficacy testing using newborn calves has relevance for evaluation of neonatal protective immunity, bovine TB control, duration of immunity studies, and as a screening tool for vaccines targeted for human testing. These attributes of the neonatal bovine model make it a useful model for screening and prioritizing vaccines before their evaluation in neonatal non-human primate models.

The region of difference 1 (*RD1*) deletion of *M. bovis* is the predominant attenuating defect in the vaccine strain developed by Calmette and Guerin in the early 1900's [i.e., *M. bovis* bacille Calmette Guerin (BCG)]. Deletion of *RD1* (i.e., Δ*RD1*) results in a loss of genes encoding early secretory antigenic target-6 kDa protein (ESAT-6) and culture filtrate protein 10 (CFP10) [10–13], as well as several other genes. Although vaccination of mice with *M. tuberculosis* Δ*RD1* provides similar protection as BCG, it has reduced safety in immune deficient mice [13]. Recent development of efficient tools for genetic manipulation of *Mycobacteria* spp. has enabled the targeted discovery of highly immunogenic attenuated live strains of *M. tuberculosis* and *M. bovis* (reviewed in [14]). Several *M. tuberculosis* auxotrophs induce significant levels of protection in mouse and guinea pig models of TB. Deletion of pantothenate biosynthesis genes from *M. tuberculosis* (i.e., *ΔpanCD*) results in an attenuated strain with limited capacity for in vivo replication while retaining similar efficacy to that of BCG in mice [15].

In the present study, a double deletion mutant of *M. tuberculosis* H37Rv strain (i.e., *ΔRD1ΔpanCD*, designated mc^{2}6030) was evaluated for efficacy in the neonatal calf model. The *RD1* deletion mimics the attenuation of BCG and the *panCD* deletion provides additional safeguards against prolonged in vivo replication, of particular importance with immune compromised hosts [13,14,16]. Responses and efficacy were compared to *M. bovis* BCG (Danish strain), the current standard for evaluation of TB vaccines.

2. Materials and methods

2.1. Animals, vaccination, and challenge procedures

Seventeen newborn Holstein bull calves were obtained from a TB-free herd in Newton, WI and housed in a biosafety level-3 (BSL-3) facility at the National Animal Disease Center, Ames, Iowa according to Institutional Biosafety and Animal Care and Use Committee guidelines. Vaccine treatment groups included: no vaccination ($n = 7$), 1.8×10^5 cfu mc^{2}6030 vaccination ($n = 6$), and 1.8×10^5 cfu BCG-Danish vaccination ($n = 4$). Selection of mc^{2}6030 dosage (i.e., 1.8×10^5 cfu) was based on prior safety and efficacy studies using mice ([16] and unpublished observations). Vaccines were administered subcutaneously at 2 weeks of age. The BCG-Danish strain was kindly provided by M.J. Brennan, United States Food and Drug Administration, Bethesda, MD. The strain of *M. bovis* used for the challenge inoculum [95-1315, USDA, Animal Plant and Health Inspection Service (APHIS) designation] was originally isolated from a white-tailed deer in Michigan, USA [17]. The challenge inoculum of this strain was prepared as described previously [18].

Challenge inoculum of 1.6×10^3 cfu in 2 ml of phosphate-buffered saline solution (PBS, 0.01 M, pH 7.2) was administered at ∼2.5 months of age by aerosol inoculation (Palmer et al., 2002). Briefly, inoculum was delivered to restrained calves by nebulization into a mask (Trudell Medical International, London, ON, Canada) covering the nostrils and mouth. Upon inspiration, inoculum was inhaled through a one-way valve into the mask and directly into the lungs via the nostrils. The process continued until the inoculum, a 1 ml PBS wash of the inoculum tube, and an additional 2 ml PBS were delivered, a process taking ∼12 min. Strict BL-3 safety protocols were followed to protect personnel from exposure to *M. bovis*.

2.2. Vaccine efficacy evaluation: bacterial recovery, mean disease score, histopathology, and lung radiographic analysis

All calves were euthanized by intravenous administration of sodium pentobarbital approximately 4 months after challenge. Tissues collected and processed for the isolation of *M. bovis* and microscopic analysis included: palatine tonsil; lung; liver; spleen; and mandibular, parotid, medial retropharyngeal, mediastinal, hepatic, mesenteric, and superficial cervical lymph nodes. Lymph nodes were sectioned at 0.5 cm intervals and examined. Following radiography, each lung lobe was sectioned at 0.5–1.0 cm intervals and examined separately. Lungs and lymph nodes (mediastinal and tracheobronchial) were evaluated using a semi-quantitative gross pathology scoring system adapted from [19]. Lung lobes (left cranial, left caudal, right cranial, right caudal, middle and accessory) were individually scored based upon the following scoring system: 0 = no visible lesions; 1 = no external gross lesions, but lesions seen upon slicing; 2 = <5 gross lesions of <10 mm in diameter; 3 = >5 gross lesions of <10 mm in diameter; 4 = >1 distinct gross lesion of >10 mm in diameter; 5 = gross coalescing lesions. Cumulative mean scores were then calculated for each entire lung. Lymph node pathology was based on the following scoring system: 0 = no necrosis or visible lesions; 1 = small focus (1–2 mm in diameter); 2 = several small foci; 3 = extensive necrosis. Data are presented as mean (±standard error) disease score for each tissue.

Tissues collected for microscopic analysis were fixed by immersion in 10% neutral buffered formalin. For microscopic examination, formalin-fixed tissues were processed by standard paraffin-embedment techniques, cut in 5 μm sections and stained with hematoxylin and eosin. Adjacent sections were cut from samples containing caseonecrotic granulomata suggestive of tuberculosis and stained by the Ziehl-Neelsen technique for identification of acid-fast bacteria. Microscopic tuberculous lesions were staged (I–IV) based on adaptations of the criteria described by [20] and [21]. Stage I (initial) granulomas are characterized by accumulations of epithelioid macrophages with low numbers of lymphocytes and neutrophils. Multinucleated giant cells may be present but necrosis is absent. Acid-fast bacilli are often absent or present in low numbers within macrophages or multinucleated giant cells. Stage II (solid) granulomas are characterized by accumulations of epithelioid macrophages surrounded by a thin connective tissue capsule. Infiltrates of neutrophils and lymphocytes may be present as well as multinucleated giant cells. Necrosis when present is minimal. Stage III (necrotic) granulomas are characterized by complete fibrous encapsulation. Necrotic cores are surrounded by a zone of epithelioid macrophages admixed with multinucleated giant cells and lymphocytes. Stage IV (necrotic and mineralized) granulomas are characterized by thick fibrous capsules, irregular multicentric granulomas with multiple necrotic cores. Necrotic cores contain foci of dystrophic mineralization. Epithelioid macrophages and multinucleated giant cells surround necrotic areas and there may be moderate to dense infiltratres of lymphocytes. Acid-fast bacilli are often present in moderate numbers primarily located within the caseum of the necrotic core. Data are presented as total and mean number of granulomas observed in each histologic lesion stage (i.e. I–IV) for lung and mediastinal lymph node sections.

For quantitative assessment of mycobacterial burden, tracheobronchial lymph nodes were removed, weighed, and homogenized in phenol red nutrient broth using a blender (Oster, Shelton, CT). Logarithmic dilutions (10^0 to 10^{-9}) of homogenates in PBS were plated in 100 μl aliquots on Middlebrook 7H11 selective agar plates (Becton Dickinson) or Middlebrook PAN (for $mc^2$6030, Teknova, Hollister, CA) and incubated for 8 weeks at 37 °C. Data are presented as mean (±standard error) cfu per gram of tissue.

To provide an additional measure of the extent of lung lesions, lung lobes were removed at necropsy and individually radiographed using a MinXray machine (Model HF-100, Diagnostic Imaging, Rapid City, SD) with 3M Asymetrix Detail Screens and Ultimate 2000 film (3M Animal Care Products, St. Paul, MN) as described [22]. Lesions were identified on digital images of scanned radiographs, outlined and measured using Image Pro Plus (Media Cybernetics, Silver Spring, MD) software (Fig. 1). Affected area was divided by total lung area then multiplied by 100 to determine percent affected lung. Using combined data from each lung lobe, results for individual animals are presented as the mean (±standard error) percentage of affected lung.

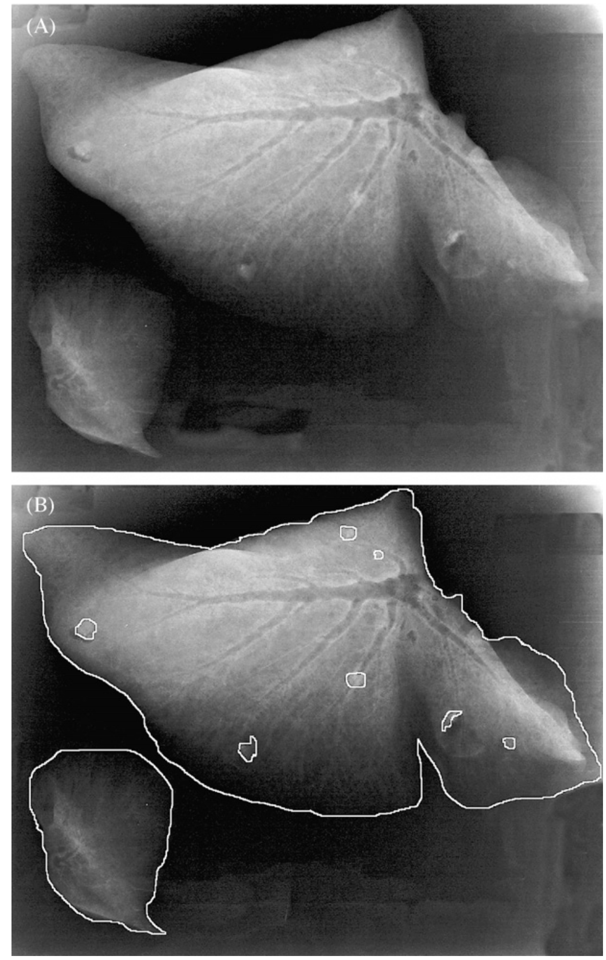

Fig. 1. Technique for identifying lung lobe margins and tuberculous lesions on radiographs for morphometry anlaysis. (A) Lung lobes were removed at necropsy and individually radiographed as described [22]. Right middle and caudal and accessory lung lobes are presented in this image. (B) Lung margins and lesions were outlined and measured using Image Pro Plus (Media Cybernetics) software.

2.3. Interferon-γ analysis

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation of peripheral blood buffy coat fractions collected into 2× acid citrate dextrose. Individual wells of 96-well round-bottom microtiter plates (Falcon, Becton-Dickinson; Lincoln Park, New Jersey) were seeded with 5×10^5 PBMC in a total volume of 200 μl per well. Medium was RPMI 1640 (GIBCO, Grand Island, New York) supplemented with 2 mM L-glutamine, 25 mM HEPES buffer, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 1% non-essential amino acids (Sigma), 2% essential amino acids (Sigma), 1% sodium pyruvate (Sigma), 50 mM 2-mercaptoethanol (Sigma), and 10% (v/v) fetal bovine sera (FBS). Wells contained medium plus 10 μg/ml

M. bovis PPD (Prionics AG, Schlieren, Switzerland), 1 μg/ml rESAT-6:CFP-10, 1 μg/ml pokeweed mitogen (PWM), or medium alone (no stimulation). After incubation for 48 h at 37 °C in 5% CO_2, culture supernatants were harvested and stored at −80 °C until thawed for analysis by an ELISA kit (Bovigam, Prionics AG). Concentrations of IFN-γ in test samples were determined by comparing absorbances of test samples with absorbances of standards within a linear curve fit. Mean IFN-γ concentrations (ng/ml) produced in 48-h cultures in response to antigen or mitogen minus concentrations in non-stimulated cultures (i.e., Δ IFN-γ) are presented.

2.4. Skin test procedures

Immediately prior to necropsy, calves received 0.1 ml (100 μg) of *M. bovis* PPD and 0.1 ml (40 μg) of *M. avium* PPD intradermally at separate clipped sites in the mid-cervical region according to guidelines described in USDA, APHIS circular 91-45-01 for the comparative cervical test. Skin thickness was measured with calipers prior to PPD administration and 72 h after injection. Balanced PPD's were obtained from the Brucella and Mycobacterial Reagents section of National Veterinary Services Laboratory, Ames, IA.

2.5. Real time rtPCR analysis

Mononuclear cell cultures were prepared as described for IFN-γ assay and incubated 16 h at 37 °C. Cells were harvested and lysed with 200 μl RLT buffer (Qiagen, Valencia, CA). RNA was isolated using an RNeasy Mini Kit (Qiagen) according to the manufacturer's directions and eluted from the column with 50 μl RNase Free water (Ambion, Austin, TX). Contaminating DNA was removed enzymatically by treating with DNA-free (Ambion) as directed by the manufacturer. Twenty microliters of RNA was reverse transcribed in a 50 μl reaction using Superscript II with 40 Units RNaseOUT and 0.25 μg Oligo$(dT)_{12–18}$ (Invitrogen, Calsbad, CA). Reverse-transcription was carried out at 42 °C for 60 min followed by 70 °C for 5 min. The resulting cDNA was stored at −80 °C until used in real-time PCR reactions. Real-time PCR was performed using SYBR Green Master Mix (Applied Biosystems, Foster City, CA) according to the manufacturer's directions. Briefly, 2.5 μl of cDNA were added to a 25 μl reaction with 1 μM of each primer. Primers were designed with Primer3 software [23] using sequences from cattle (*Bos taurus*) and PCR products were sequenced to verify the primers. All reactions were performed in triplicate and data analyzed with the 2-ΔΔCt method as described [24]. β-Actin served as the internal control and the media only (no stimulation) sample from each animal was used as the calibrator. Validation of the use of β-actin as the internal control was performed as suggested by [24]. Data are presented as mean (±standard error) gene expression to antigen stimulation relative to that of no stimulation.

2.6. Flow cytometry

Isolated PBMC cultures were prepared as described for IFN-γ analysis and incubated for 6 days. Phenotype analysis of PBMC was performed as described previously [25]. Briefly, cells were harvested and incubated with 1 μg primary monoclonal antibodies/10^6cells (mAb's; CACT116A, CD25; BAT31A, CD44; BAQ92A, CD62L; GC50A1, CD4; BAQ111A, CD8; CACT61A, γδ TCR; all obtained from VMRD, Pullman WA) at room temperature for 15 min. Cells were then washed and stained with isotype appropriate goat anti-mouse phycoeythrin- (Southern Biotechnology Associates, Birmingham, AL), allophycocyanin- (Caltag Laboratories, Burlingame, CA), or Peridinin Chlorophyll Protein- (Becton Dickinson) conjugated secondary antibodies at room temperature for 15 min. Four-color flow cytometric analyses was performed using a Becton Dickinson LSR flow cytometer. Data were analyzed with FlowJo software (Tree Star Inc., San Carlos, CA) and presented as the mean (±standard error) percent positive cells or geometric mean fluorescence intensity (mfi) for each marker.

2.7. Evaluation of antibody responses to lipoarabinomannan (LAM)

LAM-specific antibody in serum obtained at vaccination (∼14 days of age), before challenge (∼2 months of age) and postchallenge at necropsy (∼6 months of age) was quantified by ELISA. Aliquots of 50 μl LAM (3 μg/ml, kindly provided by J.T. Belisle, Department of Microbiology, Colorado State University, Fort Collins, CO) in carbonate buffer (pH 9.6) were added to wells of microtiter plates (Corning Inc., Corning, NY) and incubated at 37 °C for 1 h. Blocking was by addition of 3% bovine serum albumin (BSA, 200 μl per well) in Tris-buffered saline (TBS) at 37 °C for 1 h. After washing (3×), 50 μl calf sera diluted 1:50 in PBS was added to wells. Plates were incubated with sera for 1h at 37 °C, washed (3×), and incubated an additional 1 h at 37 °C with peroxidase-labeled goat anti-bovine IgM or IgG (10 μg/ml, 200 μl per well; Kirkegaard Perry Laboratories, Gaithersburg, MD). Plates were washed (5×) and the reaction developed by addition of 50 μl/well ABTS chromogen/substrate solution (Zymed Laboratories, Invitrogen, Carlsbad, CA). Washes were with TBS containing 0.05% Tween 20 (Sigma) in an ELISA plate washer (Skan washer, Molecular devices, Sunnyvale, CA) and absorbance was measured at 405 nm (Emax plate reader, Molecular devices, Sunnyvale, CA). Positive controls consisted of wells to which mAb 5c11 to LAM [26] were added. Negative controls consisted of wells prepared as described above but without the addition of LAM. Assays were performed in duplicate.

2.8. Statistics

Data were assessed for normality prior to statistical analysis and analyzed as a split-plot with repeated measures

ANOVA using Statview software (version 5.0, SAS Institute, Inc., Cary, NC). Fisher's protected-LSD test was applied when effects ($P < 0.05$) were detected. For antibody data, one-way ANOVA and pairwise Holm-Sidak test were used for normally distributed data and Kruskal-Wallis/Dunn's method non-normally distributed data using SigmaStat software (version 3.0, Systat Software Inc., San Jose, CA).

3. Results

3.1. Failure of mc^{2}6030 to protect calves from aerosol challenge with virulent M. bovis

Variables used to evaluate vaccine efficacy included gross pathology (i.e., mean disease scores), radiographic morphometry, mycobacterial culture, and histopathology (Table 1 and Fig. 2). Mean disease scores for lungs and lung-associated lymph nodes did not differ between mc^{2}6030-vaccinates and controls. Mean disease scores for lung-associated lymph nodes were lower ($P < 0.05$) for BCG-vaccinates than controls (Fig. 2 and Table 1). The percentage of affected lung area (i.e., radiographic morphometry data, Table 1) did not differ between mc^{2}6030-vaccinates and controls; however, there was a trend for reduced disease (pathology) in BCG-vaccinates as compared to that of mc^{2}6030-vaccinates ($P = 0.10$) and controls ($P = 0.12$).

Sections of mediastinal lymph node from non-vaccinated control calves and calves vaccinated with mc^{2}6030 contained more granulomas and more granulomas of advanced stage (III–IV) than did calves vaccinated with BCG (Table 2). No microscopic granulomas were seen in sections of lung examined from BCG vaccinated cattle, while non-vaccinated cattle and cattle vaccinated with mc^{2}6030 had lesions of all histologic stages (Table 3). Virulent *M. bovis* was cultured tracheobronchial lymph nodes (15 of 17), mediastinal lymph nodes (16 of 17), and one mesenteric lymph node from a control calf. Virulent *M. bovis* was cultured from lungs obtained from 3 of 7 controls, 2 of 6 mc^{2}6030-vaccinates, and 1 of 4 BCG-vaccinates. Tracheobronchial lymph node colonization was not affected by vaccination (Table 1). The vaccine strains (BCG and mc^{2}6030) were not isolated from tissues collected from any of the calves, including lymph nodes draining the site of vaccination. Overall assessment of vaccine efficacy utilizing gross pathology, radiographic mor-

Table 1
Comprehensive vaccine efficacy data

Treatment group	Gross pathology[a]	Radiographs[b]	Culture[c]
Control ($n = 7$)	2.43 (0.3)	1.93 (0.9)	3.04 (0.5)
BCG ($n = 4$)	0.50 (0)[†]	0.03 (0.02)[¶]	3.58 (0.3)
mc^{2}6030 ($n = 6$)	1.67 (0.2)	2.08 (0.9)	4.05 (0.2)

[a] At necropsy, tracheobronchial lymph nodes were visually evaluated for lesions based upon a scoring system adapted from [19]. Values represent mean (±standard error) mean disease scores.

[b] Lung lobes were removed at necropsy and individually radiographed. Lesions were identified on digital images of scanned radiographs, outlined, and measured (Fig. 1). Affected area was divided by total lung area then multiplied by 100 to determine percent affected lung.

[c] Tracheobronchial lymph nodes were homogenized in phenol red nutrient broth for serial dilution plate counting on Middlebrook 7H11 selective agar plates (Becton Dickinson, 8-week culture). Data are presented as mean (±standard error) cfu per gram of tissue.

[†] $P < 0.05$ as compared to controls and mc^{2}6030-vaccinates.

[¶] $P = 0.1$ as compared to mc^{2}6030-vaccinates.

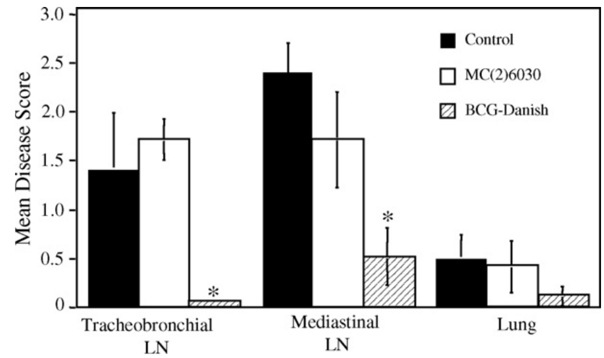

Fig. 2. Gross pathology. Lungs were evaluated based upon the following scoring system: 0 = no visible lesions; 1 = no external gross lesions, but lesions seen upon slicing; 2 = <5 gross lesions of <10 mm in diameter; 3 = >5 gross lesions of <10 mm in diameter; 4 = >1 distinct gross lesion of >10 mm in diameter; 5 = gross coalescing lesions. Scoring of lymph node pathology was based on the following system: 0 = no necrosis or visible lesions; 1 = small focus (1–2 mm in diameter); 2 = several small foci; 3 = extensive necrosis. Values represent mean (±standard error) pathology scores, ($n = 7$, controls; $n = 6$, mc^{2}6030; $n = 4$, BCG). (*) Differs from controls, $P < 0.05$.

Table 2
Histologic evaluation of lung-associated lymph node (i.e., mediastinal)

Treatment	Histologic stage[a]				
	I	II	III	IV	Total[b]
Controls ($n = 7$)	9.9	3.6	7.6	3.6	23.1 (5.3)
BCG ($n = 4$)	3.3	1.5	1.0	1.0	6.7 (3.5)[†]
mc^{2}6030 ($n = 6$)	3.5	2.2	5.2	1.7	12.2 (4.4)

[a] Microscopic tuberculous lesions were staged (I–IV) based on adaptations of the criteria described by [20] and [21]. Disease severity progresses from stage I to IV. The number of granulomas in each histologic stage per section of mediastinal LN was enumerated.

[b] Mean (±standard error) number of granulomas (i.e., regardless of stage) detected on a section of mediastinal lymph node for each vaccine treatment.

[†] $P < 0.05$ as compared to controls.

Table 3
Histologic evaluation of lung

Treatment	Histologic stage[a]				
	I	II	III	IV	Total[b]
Controls ($n = 7$)	0.9	0.3	0.9	0.4	2.4 (0.9)
BCG ($n = 4$)	0	0	0	0	0 (0)[¶]
mc^{2}6030 ($n = 6$)	0.3	0.2	0.5	0.2	1.2 (0.7)

[a] Microscopic tuberculous lesions were staged (I–IV) based on adaptations of the criteria described by [20] and [21]. Disease severity progresses from stage I to IV. The number of granulomas in each histologic stage per section of lung was enumerated.

[b] Mean number of granulomas (i.e., regardless of stage) detected on a section of lung for each vaccine treatment.

[¶] $P = 0.1$ as compared to controls.

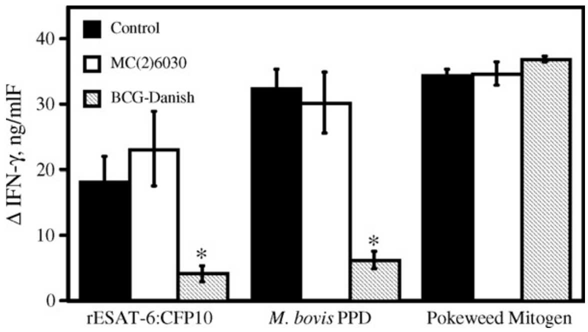

Fig. 3. Interferon-γ responses. Blood mononuclear cells were isolated from cattle ∼3 months after challenge with virulent *M. bovis* (∼5 months after vaccination) and cultured with 10 μg/ml *M. bovis* PPD (Prionics AG), 1 μg/ml rESAT-6:CFP-10, 1 μg/ml pokeweed mitogen (PWM), or medium alone (no stimulation) for 48 h. Interferon-γ concentrations in culture supernatants were quantified by ELISA. Values represent mean (±standard error) responses to stimulation (i.e., antigen or PWM) minus the response to media alone ($n = 7$, controls; $n = 6$, mc^{2}6030; $n = 4$, BCG). Responses to PWM are presented to indicate a general responsiveness of the cell population to polyclonal stimulation (i.e., a positive control). (*) Differs from controls, $P < 0.05$.

phometry, and mycobacterial culture indicated that neonatal vaccination with mc^{2}6030 failed to protect calves from low dose virulent *M. bovis* aerosol challenge (Table 1). Although BCG vaccination afforded protection from disease it did not reduce colonization of assayed tissues.

3.2. Reduced in vitro recall responses are positive prognostic indicators of vaccine efficacy

Three months after challenge and approximately 5 months after vaccination, antigen-specific IFN-γ, iNOS, IL-4, and MIP1-α responses of PBMC from BCG-vaccinates were lower than responses of cells from control and mc^{2}6030-vaccinates (Fig. 3 and Table 4). In contrast, cutaneous delayed type hypersensitivity (Fig. 4) and TNF-α (Table 4) responses evaluated concurrently were not affected by vaccination and FoxP3 responses were increased in BCG vaccinates (Table 4). IL-5 expression in antigen-stimulated cells from controls exceeded expression by cells from BCG- and mc^{2}6030-vaccinates (Table 4).

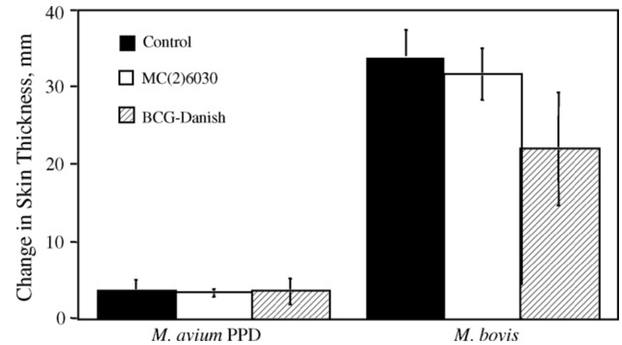

Fig. 4. Comparative cervical skin test. Immediatley prior to necropsy, animals received 0.1 ml (100 μg) of *M. bovis* PPD and 0.1 ml (40 μg) of *M. avium* PPD in the mid-cervical region according to guidelines described in USDA, APHIS circular 91-45-01. Responses are presented as mean (±standard error) skin thickness to purified protein derivative (PPD, mycobacterial origin indicated) at 72 h after injection minus the skin thickness prior to injection ($n = 7$, controls; $n = 6$, mc^{2}6030; $n = 4$, BCG). Responses did not differ between vaccine treatment groups.

Three months after challenge, stimulation of PBMC with mycobacterial antigens resulted in increased percentages of CD4+ cell ($P < 0.0001$) and decreased percentages of CD8+ cell ($P < 0.05$) in PBMC cultures (Table 5). Percentages of CD4+ cells harvested from 6 d PBMC cultures differed ($P < 0.05$) between each of the vaccine treatment groups [i.e., mc^{2}6030-vaccinates (10.2 ± 1.2) > controls

Table 5
Alterations in T cell subset composition upon in vitro antigen stimulation

Type of stimulation	CD4+[a]	CD8+	γδ TCR+
No stimulation ($n = 17$)	3.8 (0.4)	11.6 (1.0)	17.5 (1.7)
rESAT-6:CFP10 ($n = 17$)	10.2 (1.2)**	9.0 (0.9)*	15.4 (1.5)
M. bovis PPD ($n = 17$)	10.0 (0.9)**	8.7 (0.5)*	18.3 (1.6)

[a] Blood mononuclear cells were isolated from cattle ∼3 months after challenge with virulent *M. bovis* (∼5 months after vaccination) and cultured with 10 μg/ml *M. bovis* PPD (Prionics AG), 1 μg/ml rESAT-6:CFP-10, or medium alone (no stimulation). After 6 days, cells were harvested and analyzed by flow cytometry for T cell subset composition. Data are presented as the mean (±standard error) percent positive cells in PBMC cultures according to in vitro stimulation, irrespective of vaccine treatment. Differs (*$P < 0.05$, **$P < 0.01$) from non-stimulated cultures (i.e., vertical comparisons).

Table 4
Gene expression by *M. bovis* PPD stimulated blood mononuclear cells

Treatment group [a]	iNOS	IL-4	IL-5	MIP1-α	TNF-α	FoxP3
Control ($n = 7$)	7.8 (1.1)	5.9 (1.4)	264.0 (105.8)[†]	3.1 (1.0)	4.0 (0.8)	1.8 (0.9)
BCG ($n = 4$)	4.1 (0.4)[¶]	2.1 (0.3)[‡]	4.3 (2.8)	1.4 (0.1)[‡‡]	2.5 (0.3)	48.3 (26.5)[‡‡‡]
mc^{2}6030 ($n = 6$)	7.2 (0.8)	7.4 (2.0)	20.2 (7.5)	3.5 (0.9)	4.1 (0.7)	12.2 (10.2)

[a] Blood mononuclear cells were isolated from cattle ∼3 months after challenge with virulent *M. bovis* (∼5 months after vaccination) and cultured with either medium plus 10 μg/ml *M. bovis* PPD (Prionics AG) or medium alone (no stimulation). After 16 h of culture, cells were harvested, RNA isolated, and gene expression evaluated by real time RTPCR as described in the Methods. Data are presented as mean (±standard error) gene expression to *M. bovis* PPD stimulation relative to that of no stimulation. Similar responses were detected by PBMC in response to rESAT-6:CFP10 stimulation (data not shown).

[¶] $P < 0.05$ as compared to control and mc^{2}6030-vaccinates.

[†] $P < 0.05$ as compared to BCG- and mc^{2}6030-vaccinates.

[‡] $P < 0.05$ as compared to mc^{2}6030-vaccinates and $P = 0.07$ as compared to controls.

[‡‡] $P < 0.1$ as compared to mc^{2}6030-vaccinates.

[‡‡‡] $P < 0.05$ as compared to controls and $P = 0.06$ as compared to BCG-vaccinates.

(7.8 ± 0.9) > BCG-vaccinates (5.0 ± 1.2)]. Percentages of γδ TCR+ cells were not affected by type of stimulation in vitro; however, increased percentages of γδ TCR+ cells were detected in cultures from BCG vaccinates (21.1 ± 2.1) as compared to control (15.0 ± 1.1) and mc^{2}6030-vaccinates (16.8 ± 1.5) (P = 0.01 and 0.07, respectively). As previously reported [25], stimulation with mycobacterial antigen resulted in increased percentages (and associated changes in mfi) of CD25+ and CD44+ cells and decreased percentages of CD62L+ cells in PBMC cultures (P < 0.002), irrespective of vaccine treatment (data not shown). Alterations in phenotype indicative of lymphocyte activation (i.e. increased CD25 and CD44 expression and reduced CD62L expression) were most prominent in PBMC cultures from mc^{2}6030-vaccinates and controls. Effects of vaccine treatment on antigen [i.e., rESAT-6:CFP10 (Fig. 5A) and *M. bovis* PPD (Fig. 5B)] induced responses are shown in Fig. 5. A diminished activation phenotype of fewer CD25+ and CD44+ cells and greater CD62L+ cells was evident in antigen-stimulated PBMC cultures from BCG-vaccinates as compared to control and mc^{2}6030-vaccinates.

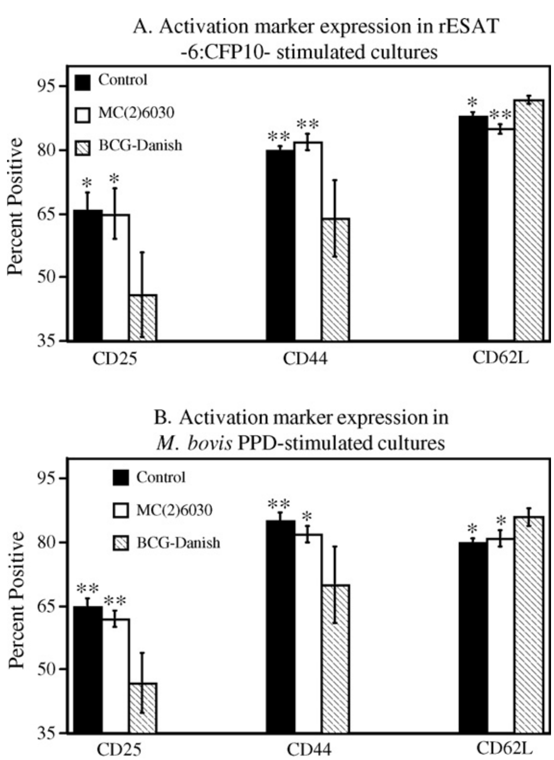

Fig. 5. Percent CD25+, CD44+, and CD62L+ cells in rESAT-6:CFP10-stimulated (A) and *M. bovis* PPD-stimulated (B) cultures. Mononuclear cells were isolated by density gradient centrifugation of buffy coat fractions and cultured with 1 μg/ml rESAT-6:CFP10 or 1 μg/ml *M. bovis* PPD. After 6 days of culture, cells were harvested for each individual animal according to treatment and stained with mAb's to cell surface markers for analysis by flow cytometry. Data are presented as mean (±standard error) percent expression of activation markers. Asterisks indicate differences (**P < 0.1, *P < 0.05) in PBMC cultures from control and mc^{2}6030-vaccinates as compared to BCG-vaccinates.

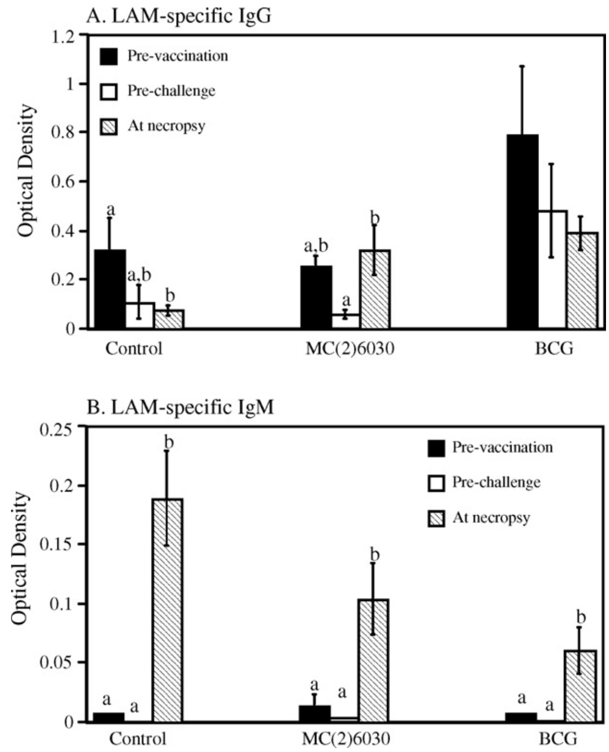

Fig. 6. Serum IgG (A) and IgM (B) to LAM. Serum was obtained from calves at 2 weeks of age (pre-vaccination), ∼2 months of age (pre-challenge), and ∼4 months after challenge (at necropsy) and evaluated for LAM-specific antibody by ELISA. Data are presented as mean (±standard error) absorbance. Bars with differing letter designation (i.e., a versus b) are different (P < 0.05) within vaccine treatment.

3.3. *Antibody responses to lipoarabinomannan*

With the exception of one calf from the unvaccinated group, LAM-specific IgG was detected in the serum from all calves prior to immunization (∼2 weeks of age) (Fig. 6A), indicating colostral transfer of IgG reactive to LAM as demonstrated previously [6]. In general, LAM-specific IgG decreased throughout the study indicating decay in colostrum-derived antibody and blocking of an IgG response to both vaccination and subsequent infection. The exception was an increase (P < 0.05) in LAM-specific IgG in serum from mc^{2}6030-vaccinates after challenge with virulent *M. bovis* as compared to responses immediately pre-challenge (i.e., after vaccination with mc^{2}6030). In contrast to IgG, LAM-specific IgM was not detected prior to challenge and increased in all groups upon challenge (Fig. 6B).

4. Discussion

Vaccination with mc^{2}6030 failed to protect calves from virulent *M. bovis* aerosol challenge, despite a proven record of efficacy with immune-competent and immune-deficient strains of mice [16]. Similarly, vaccination trials with mc^{2}6030 failed to protect cynomolgus monkeys from virulent *M. tuberculosis* (Larsen et al., unpublished observations).

Experimental challenge with a strain of *M. tuberculosis* virulent to mice, monkeys, and humans did not elicit disease in cattle (Vordermeier and Hewinson, personal communication). For cattle, attenuated *M. tuberculosis* mutants may be less immunogenic as compared to those produced on a virulent *M. bovis* or BCG background strain. Thus, cattle may not be as useful as other models (e.g., monkeys) for the study of vaccine efficacy using *M. tuberculosis* mutants. Further studies are required to directly compare immunogenicity and virulence of *M. tuberculosis* versus *M. bovis* background mutants in cattle. Similar findings with cynomolgus monkey and neonatal calf trials, however, may be indicative of other, non-determined causes of vaccine failure such as inappropriate dose.

In contrast to $mc^2 6030$, BCG-vaccinated cattle exhibited both limited disease dissemination and disease severity as evidenced by decreased lesion scores, decreased pulmonary involvement (radiographic morphometry) and fewer granulomas of advanced histologic stage in mediastinal lymph nodes and lungs. Development of fewer granulomas with less necrosis likely limits transmission, as it is later stage granulomas with extensive necrosis and large numbers of acid-fast bacilli that facilitate disease dissemination within individual hosts and between individuals. Colonization of tracehobronchial lymph nodes with virulent *M. bovis* was unaffected by vaccine treatment, suggesting that BCG vaccination reduced TB-associated pathology without limiting colonization.

Comparison of immune responses to mycobacterial antigen after virulent challenge demonstrated unique differences in the response associated with prior vaccine treatments. In general, BCG-vaccinates had a diminished activation profile as compared to $mc^2 6030$-vaccinates and controls. BCG-vaccinates had reduced antigen-specific IFN-γ, iNOS, IL-4, and MIP1-α responses; reduced expansion of CD4+ cells in culture; and a diminished activation profile of decreased percentages of CD25+ and CD44+ cells and increased percentages of CD62L+ cells. Thus, a reduced immune stimulation profile was associated with administration of a disease limiting vaccine (i.e., BCG). These findings are in agreement with [19] and [27] demonstrating that a low response to ESAT-6 upon experimental challenge is a positive prognostic indicator for vaccine efficacy.

In cattle, T cell responses at the clonal level are biased to a Th1 response with few if any Th2 clones expressing IL-4 independent of IFN-γ [28,29]. The host response to mycobacterial infection relies, in part, on the production of IFN-γ by Th1 and CD8+ effector T cells in addition to cytolytic mechanisms for clearance of bacilli infected cells. This potentially harmful inflammatory response may be counter balanced by FoxP3 expressing $CD4^+CD25^+$T regulatory cells [30] or IL-13-/IL-4-expressing cells [31]. In the present study, high expression levels of FoxP3 and low expression levels of IL-4 and IL-5 were associated with a disease limiting vaccine (BCG) as compared to non-protected animals (i.e., non- and $mc^2 6030$-vaccinates). In contrast, $CD4^+CD25^+FoxP3^+$ T regulatory cells increase in humans with active TB and suppress IFN-γ and IL-10 production, thereby, contributing to disease pathogenesis [30,32]. FoxP3 expression is increased in PBMC from humans with TB and correlates to increased numbers of circulating $CD4^+CD25^+$ T-regulatory cells [33]. Definitive characterization of the role of T regulatory cells in vaccine-induced immunity of cattle to TB will require further phenotypical and functional characterization of these populations.

LAM was chosen as the target antigen for evaluation of the antibody response because it is a major surface component of mycobacteria involved in the immunopathogenesis of tuberculosis including apoptosis, inhibition of phagosomal maturation, and macrophage interferon-γ signaling (reviewed in [34]). In the present study, LAM-specific IgG was detected in the serum from 2 weeks old calves. Although not statistically significant and of questionable relevance, LAM-specific IgG levels were numerically greater in BCG vaccinates as compared to other animals. IgG to mycobacterial antigens is transferred from the dam to the calf via colostrum with levels correlating to the immunologic experience of the dam ([6] and Waters/Nonnecke, unpublished observations). Exposure of dams to ubiquitous non-tuberculous, environmental mycobacteria elicits IgG reactive to LAM that is then transferred to the calf. In contrast, LAM-specific IgM was not detected prior to challenge (Fig. 6B), indicating a lack of maternal transfer of this isotype and no response to vaccination. As previously described [6], maternal LAM-specific IgG blocks production of antibody to LAM in the neonatal calf. Experimental challenge with *M. bovis*, on the other hand, induced significant levels of LAM-specific IgM. By this time (i.e., ~2.5 months of age), it is likely that the levels of IgG had waned to a level insufficient to block a response to mycobacterial infection; thus, as would be expected, an early IgM response to infection was elicited (Fig. 6B).

In summary, the calf sensitization and challenge model provides an informative screen for candidate tuberculosis vaccines before their evaluation in costly non-human, primates.

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